Novel microtubule-targeting agents, pyrrolo-1,5-benzoxazepines, induce cell cycle arrest and apoptosis in prostate cancer cells.

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Running title: PBOXs induce arrest and apoptosis in prostate cancer cells

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Abstract

Advanced hormone-refractory prostate cancer is associated with poor prognosis and limited treatment options. Members of the pyrrolo-1,5-benzoxazepine (PBOX) family of compounds exhibit anti-cancer properties in cancer cell lines (including multi-drug resistant cells), *ex vivo* patient samples and *in vivo* mouse tumour models with minimal toxicity to normal cells. Recently, they have also been found to possess anti-angiogenic properties *in vitro*. However, both the apoptotic pathways and the overall extent of the apoptotic response induced by PBOX compounds tends to be cell-type specific. Since the effect of the PBOX compounds on prostate cancer has not yet been elucidated, the purpose of this study was to investigate if PBOX compounds induce anti-proliferative effects on hormone-refractory prostate cancer cells. We examined the effect of two representative PBOX compounds, PBOX-6 and PBOX-15, on the androgen-independent human prostate adenocarcinoma cell line, PC3. PBOX-6 and -15 displayed anti-proliferative effects on PC3 cells, mediated initially through a sustained G_2/M arrest. G_2/M arrest, illustrated as DNA tetraploidy, was accompanied by microtubule depolymerisation and phosphorylation of anti-apoptotic proteins Bcl-2 and Bcl-x_L and the mitotic spindle checkpoint protein BubR1. Phosphorylation of BubR1 is indicative of an active mitotic checkpoint and results in maintenance of cell cycle arrest. G_2/M arrest was followed by apoptosis illustrated by DNA hypoploidy and PARP cleavage and was accompanied by degradation of BubR1, Bcl-2 and Bcl-x_L. Furthermore, sequential treatment with the CDK1-inhibitor, flavopiridol, synergistically enhanced PBOX-induced apoptosis. In summary, this *in vitro* study indicates that PBOX compounds may be useful alone or in combination with other agents in the treatment of hormone-refractory prostate cancer.

Introduction

Prostate cancer is the leading cause of cancer and the second leading cause of cancer-related deaths among men in Europe and the USA (1). Early diagnosis following widespread PSA screening has resulted in most patients presenting with localized tumours. These patients generally exhibit good survival rates following radiotherapy or surgery. However, a number of patients still progress to or are diagnosed with locally advanced or metastatic tumours. While androgen-deprivation therapy can slow the development of advanced metastatic prostate cancer, most tumours will still progress within a few years to an androgen-independent state (2). At this stage, treatment options become limited. The current treatment of choice for patients with advanced hormone-refractory prostate cancer is docetaxel-based chemotherapy. While these treatments significantly improve the overall survival of patients (3,4), the prognosis still remains poor for patients presenting with advanced stage prostate cancer. Therefore, the development of improved treatments and combinations are required.

Our research group have established that some members of the pyrrolo-1,5-benzoxazepine (PBOX) family of compounds are capable of inducing apoptosis in cancerous cell lines derived from both haematological malignancies (promyelocytic leukaemia HL60 cells, Jurkat T-lymphoma cells, Hut-78 lymphoma cells, T cell leukaemia CEM cells and chronic myeloid leukaemia (CML) K562 cells) and solid tumours (breast carcinoma MCF-7 cells and ovarian A2780 cells) (5-10). These results are supported by studies revealing impaired growth of tumours in a mouse 4T1 breast carcinoma tumour model (11), in a CML mouse model (12) and apoptosis in *ex vivo* CML patient samples (12, 13). Interestingly, these compounds exert only minimal toxicity to normal peripheral blood mononuclear cells (PBMCs) (8) or bone marrow cells (13). In addition we have also found that PBOXs displayed anti-angiogenic properties *in vitro* (10). An added advantage of PBOX compounds over many clinically used

anti-cancer agents such as docetaxel is that they do not appear to be substrates of the ABC-transporters, P-glycoprotein and BCRP, since they are capable of inducing apoptosis in multidrug-resistant cancer cells expressing these transporters with similar efficacy as in transporter-negative drug-sensitive cells (14). The PBOX compounds exert their effects by binding to an uncharacterised site on tubulin resulting in microtubule depolymerisation (15). Our studies to date indicate that the mechanistic pathways downstream of tubulin depolymerisation and the overall extent of the apoptotic response tends to be cell-type specific. For example, we have shown that caspases are absolutely required for PBOX-induced apoptosis in promyelocytic leukaemia HL60 cells (5) but not in CML K562 cells (6). We have also elucidated that in response to PBOX-treatment, some cell types, such as K562 cells, exhibited a sustained mitotic arrest while other cell types, such as HL60 cells, arrested transiently and then proceeded to undergo apoptosis (16).

The effect of PBOX compounds on prostate cancer has not yet been documented. Therefore, in this study we examined the effect of two representative PBOXs, PBOX-6 and PBOX-15 (Fig. 1) on androgen-independent prostate cancer PC3 cells. We show that these compounds are capable of inhibiting proliferation of PC3 cells. This response was manifested by G_2/M arrest followed by apoptosis. These events coincide with destabilisation of the microtubular network and phosphorylation of the anti-apoptotic proteins Bcl-2 and Bcl-x_L and the mitotic spindle checkpoint protein BubR1. Furthermore we also established that sequential treatment with CDK1 inhibitor, flavopiridol, synergistically enhanced PBOX-induced apoptosis in PC3 cells. Together with our previous findings these results indicate that PBOX compounds may prove to be useful agents in the treatment of hormone-refractory prostate cancer and therefore are worthy of further investigation alone or in combination with other agents as potential treatments for advanced prostate cancer.

Materials and methods

Unless otherwise stated, chemicals were obtained from Sigma-Aldrich (Poole, Dorset, UK), antibodies from Merck Biosciences (Nottingham, UK) and tissue culture vessels were sourced from Greiner Bio-One GmbH (Frickenhausen, Germany).

Cell Culture

Originally established from a bone metastasis of a grade IV human prostatic adenocarcinoma, PC3 cells, were obtained from the European Collection of Cell Cultures (Salisbury, UK). PC3 cells were maintained in Roswell Park Memorial Institute (RPMI)-1640 medium enhanced with GlutaMAX-I and supplemented with 10% foetal bovine serum (FBS), 50 units/ml penicillin and 50 µg/ml streptomycin (all from Gibco, Invitrogen, Carlsbad, CA, USA). Cells were maintained in a humidified incubator at 37°C in 5% CO_2 and subcultured by trypsinisation upon reaching ~80% confluency.

Reagents

The pyrrolo-1,5-benzoxazepine compounds, 7-[(N,N-dimethylcarbamoyl)oxy]-6-(naphth-1-yl)pyrrolo[2,1-d][1,5]benzoxazepine (PBOX-6), 4-acetoxy-5-(1-(naphthyl)naphtho[2,3-*b*]pyrrolo[2,1-*d*][1,4]oxazepine (PBOX-15) and pyrrolobenzoxazepine 7-[(diethylcarbamoyl)oxy]-6-p-toylpyrrolo[2,1-d][1,5]benzoxazepine (PBOX-21) were synthesised as described previously (17) and dissolved in ethanol. The chemical structures of PBOX-6 and PBOX-15 are shown in Figure 1. Paclitaxel and flavopriadol were dissolved in DMSO while vincristine was dissolved in water. Once reconstituted in the relevant solvent, all compounds were stored at –20°C with the exception of flavopiridol which was stored at 4°C.

Cell proliferation

Cell proliferation was monitored using AlamarBlue™ dye (BioSource, Invitrogen, Carlsbad, CA, USA) which changes to a fluorescent state in the reduced environment of living cells. Cells (20,000 cells/well) were seeded on 96-well plates for 24 h and then treated with a range of concentrations of PBOX-6 or PBOX-15 for 72 h. AlamarBlue™ (final concentration 10% (v/v)) was added and incubated at 37°C. Fluorescence was measured at an excitation wavelength of 544nm and an emission wavelength of 590nm using a SpectraMax Gemini spectrofluorometric plate reader (Molecular Devices, Sunnyvale, CA). The results were expressed as the percentage cell viability relative to vehicle-treated control cells (100%). Dose-response curves were plotted and IC_{50} values (concentration of drug resulting in 50% reduction in cell viability) were obtained using Prism GraphPad 4.

Determination of DNA content

Following treatment, adherent and non-adherent cells were harvested by trypinisation and centrifugation at 800 g for 10 min. Cell pellets were resuspended in 200 µl PBS and fixed by a drop-wise addition of 2 ml of ice-cold 70% (v/v) ethanol/PBS while gently vortexing. Following overnight fixation at -20°C, the cells were centrifuged with 5 µl FBS to remove the ethanol and resuspended in PBS supplemented with 0.5 mg/ml RNase A and 0.15 mg/ml propidium iodide (PI). Cells were incubated in the dark at 37°C for 30 min. The PI fluorescence was measured on a linear scale using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). Data collections (10,000 events per sample) were gated to exclude cell debris and cell aggregates. PI fluorescence was proportional to the amount of DNA present in each entity and therefore indicated the stage of the cell cycle. Cells in G_0/G_1 were diploid (2N DNA content), cells in the S phase had DNA contents between 2N and 4N, cells in G_2/M were tetraploid (4N DNA content), while apoptotic cells were hypoploid and

contained <2N DNA. All data was recorded and analysed using the CellQuest software (Becton Dickinson, San Jose, CA, USA).

Microtubule staining by indirect immunofluorescence

PC3 cells (80,000 cell/ml), cultured on BD Falcon™ 4-chamber glass slides (BD Biosciences, San Jose, CA, USA) for 24 h were treated for 16 h. The cells were fixed in 100% methanol at -20°C (10 min). The cells were then incubated in the following solutions, each incubation preceded by washing in PSB (Oxoid Ltd., Hampshire, UK): blocking solution (5% (w/v) BSA dissolved in 0.1% (v/v) Triton X-100 in PBS) (30 min), monoclonal anti-α-tubulin antibody (Merck Biosciences, Nottingham, UK) (1 h), fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse antibody (DakoCytomation, Glostrup, Denmark) (1 h) and 0.2 μg/ml propidium iodide (2 min). The chamber partitions were removed from the slides and anti-quenching solution (2 μg/ml *p*-phenylenediamine in 50:50 glycerol to PBS solution) was applied to the surface of each slide and coverslips mounted. The organisation of the microtubule network (green) and the cellular DNA (red) was visualised under a 60X oil-emersion lens using an Olympus IX81 Fluorescent Microscope (Olympus Corporation, Tokyo, Japan).

Analysis of protein expression and cleavage by Western blotting

Cells were harvested in whole cell lysis buffer containing 62.5 mM Tris (pH 6.8), 2% (w/v) SDS, 10% (v/v) glycerol, 0.00125% (w/v) bromophenol blue and 50 mM DTT. The samples were then sonicated briefly and denatured at 65°C for 10 min before separation of proteins on a polyacrylamide gel and transfer to PVDF membrane. The PVDF transfers were probed with primary antibodies generated against PARP, BubR1 (BD Transduction Laboratories, Cowley, UK), Bcl-2, Bcl-x_L or β-actin. This was followed by incubation with a horseradish

peroxidase-conjugated secondary antibody (Promega, Madison, WI, USA). Protein expression was visualised by enhanced chemiluminescence.

Statistical analysis

Results were presented as mean ± S.E.M. The statistical analysis of experimental data was performed using the computer program Prism GraphPad 4. P-values were determined using a two-tailed Student's paired t-test. A value of $P < 0.05$ was considered to be significant.

Analysis of drug interactions

Drug interactions were determined by median dose effect analysis using the Calcusyn (Biosoft, Cambridge, UK). This method is based on the drug effect equation of Chou and Talalay and can determine the degree of synergism or antagonism between two compounds by generating a combination index (CI) value. CI values of <1, =1 and >1 indicate synergism, an additive effect and antagonism respectively (18).

Results

PBOX compounds reduced prostate cancer cell proliferation.

Hormone-refractory prostate adenocarcinoma PC3 cells were treated for 72 h with a range of concentrations of PBOX-6 or PBOX-15. Cell proliferation was measured by AlamarBlue assay. PBOX-6 and -15 reduced proliferation of PC3 cells in a dose-dependent manner. PBOX-15 was found to be the more potent of the two analogues. IC_{50} values (concentration of drug resulting in 50% reduction in cell viability) were 5.8 μM and 0.37 μM for PBOX-6 and -15 respectively (Fig. 2A). The concentrations of drugs used for the remaining experiments were chosen to reflect the IC_{50} values obtained by this cell proliferation assay.

PBOX compounds induced G_2/M arrest and apoptosis in prostate cancer cells.

Flow cytometric analysis of propidium iodide staining allowed us to measure the DNA content of PC3 cells. PBOX-6 (10 μM) and -15 (0.5 μM) induced statistically significant increases in the percentage of PC3 cells found in the G_2/M phase (4N DNA content) of the cell cycle (Fig. 2B) which peaked for both compounds after 16 h. In comparison to vehicle-treated PC3 cells, where only 26.6 ± 1.6% of cells were found in the G_2/M phase, 72.9 ± 2.5% of cells were G_2/M arrested following 16 h treatment with PBOX-6 (**P=0.0058) or 68.0 ±4.6% for PBOX-15 (*P=0.0161). The levels of G_2/M arrest dropped at later time points to 32.6 ± 1.7% and 44.9 ± 4.1% for PBOX-6 and -15 respectively after 72 h. This decline in the levels of G_2/M arrest corresponded with significant increases in the amount of apoptotic cells (distinguished as cells displaying DNA hypoploidy, <2N DNA content) (Fig. 2C) with levels of apoptosis rising from 1.4 ± 0.1% for vehicle-treated cells to 23.2 ± 1.8% for 10 μM P6 (**P-value = 0.0068) and 18.2 ± 1.8% for 0.5 μM PBOX-15 (*P-value=0.0133) after 72 h. These increases in DNA hypoploidy were accompanied by one of the key indicators of apoptosis, cleavage of the DNA repair enzyme PARP (Fig. 2D).

PBOX compounds resulted in destabilisation of the microtubule network in prostate cancer cells.

PC3 cells were treated with PBOX-6 or -15 for 16 h (time of maximum G_2/M arrest). As a positive control, cells were also treated with the known tubulin polymeriser, paclitaxel and the tubulin depolymeriser, vincristine. As a negative control, cells were treated with a non-apoptotic member of the PBOX family, the G_1-arresting compound PBOX-21 (19, 20). Immunofluorescent staining was used to detect morphological changes in the microtubule network, such as, alterations in organisation and arrangement (Fig. 3). In normal cells, the microtubule network is organised as cytoplasmic tubulin filaments radiating from a central point to the periphery. Cells treated with vehicle alone (0.5% ethanol) displayed this typical tubulin morphology. Exposure of cells to the tubulin polymerising agent, paclitaxel, resulted in a highly concentrated accumulation of filaments into dense peripheral bundles indicative of microtubule stabilisation. The tubulin depolymerising agent, vincristine, resulted in diffuse tubule staining with no definition of structure caused by microtubule disassembly. While no change in microtubule structure was evident following treatment with the lower doses of 1 μM PBOX-6 or 50 nM PBOX-15, gross morphological changes in the tubulin cytoskeleton typical of depolymerising agents and visualised as diffuse tubule staining were observed when cells were treated with 10 μM PBOX-6 or 0.5 μM PBOX-15. As expected, exposure to the G_1-arresting agent, PBOX-21 (25 μM), did not disrupt the microtubular network of PC3 cells and indicated that interference to the normal organisation of the microtubules is required for G_2/M arrest in PC3 cells.

PBOX compounds induced phosphorylation followed by degradation of BubR1 in prostate cancer cells.

Treatment of PC3 cells with 10 μM PBOX-6 or 0.5 μM PBOX-15 resulted in a time-dependent phosphorylation of the mitotic spindle checkpoint protein BubR1 represented in Western blotting as the presence of a slower migrating band (Fig. 4A). Small amounts of phosphorylated BubR1 were evident as early as 4 h post treatment and became most prominent after 16 and 24 h. This hyperphosphorylation of BubR1 correlated to G_2/M arrest and indicated an active mitotic checkpoint. At the later time point of 48 h, levels of BubR1 decreased with very little BubR1 protein detectable after 72 h, corresponding to a decline in G_2/M and an increase in apoptosis. β-Actin was used as a loading control.

PBOX compounds induced phosphorylation followed by degradation of Bcl-2 and Bcl-x_L in prostate cancer cells.

Treatment with 10 μM PBOX-6 or 0.5 μM PBOX-15 for 16 h resulted in phosphorylation of the anti-apoptotic proteins Bcl-2 and Bcl-x_L, again evidenced by a slower migrating band on a Western blot (Fig.4B). Levels of Bcl-2 began to decrease 24 h post-treatment with no Bcl-2 detectable after 72 h treatment. Phosphorylation of Bcl-x_L was more sustained, since after 24 h the phosphorylated forms of Bcl-x_L were still prominent. However, levels of Bcl-x_L had declined by 48 h post-treatment. These results indicate that Bcl-2 and Bcl-x_L are inactivated in prostate cancer cells by PBOX compounds. Again β-Actin was used as a loading control.

The CDK-1 inhibitor, flavopiridol, synergistically enhanced PBOX-induced apoptosis in prostate cancer cells.

To investigate if the CDK-1 inhibitor flavopiridol could enhance the apoptotic efficacy of PBOX compounds in hormone-refractory prostate cancer cells, we treated PC3 cells with either vehicle (0.5% EtOH), PBOX-6 (1, 5, 10 μM) or PBOX-15 (0.1, 0.25, 0.5 μM) for 18 h followed by treatment with either flavopiridol (0.1, 0.25, 0.5 u μM) for 24 h or fresh media

(control). Apoptosis was quantified by flow cytometric analysis as the number of cells with hypoploid DNA content (<2N DNA). Treatment with vehicle for 18 h followed by treatment with flavopiridol (0.5 μM) for 24 h resulted in a low level of apoptosis 7.2 ± 0.2% compared to 2.7 ± 1.0% with vehicle alone (Fig.5A). Treatment with PBOX-6 (5 μM) for 18 h followed by incubation in fresh media resulted in 11.9 ± 3.0% apoptosis, while sequential treatment with flavopiridol (0.5 μM) for 24 h after the PBOX-6 incubation step resulted in an increase in apoptosis to 18.2 ± 1.5% (data not shown). PBOX-6 (10 μM) for 18 h followed by incubation in fresh media resulted in 12.2 ± 1.4% apoptosis whilst the same concentration of PBOX-6 followed by exposure to flavopiridol caused a statistically significant increase in apoptosis to 28.8 ± 1.7% (*P=0.0264) (Fig. 5A). Similarly, sequential treatment with flavopiridol increased 0.25 μM PBOX-15-induced apoptosis from 12.9 ±2.8% for PBOX-15 alone to 26.1 ± 3.5% for the combination (data not shown) and caused a statistically significant increase in apoptosis from 15.1 ± 0.5% for 0.5 μM PBOX-15 alone to 32.4 ± 2.3% for the combination (*P=0.0274) (Fig. 5A).

To determine if the enhancement in apoptosis we observed between flavopiridol and PBOX-6 or -15 could be considered synergistic, we used the CalcuSyn program to perform a median dose analysis of our results. We found that flavopiridol (0.5 μM) in combination with 5 μM or 10 μM PBOX-6 was considered synergistic as they displayed combination index (CI) values of less than 1 (CI = 0.381 or 0.335 respectively) (Fig. 5B). Similarly, flavopiridol in combination with 0.25 μM or 0.5 μM PBOX-15 was found to be synergistic with CI values 0.300 and 0.433 respectively (Fig. 5C).

The lower concentrations of 1 μM PBOX-6 and 0.1 μM PBOX-15 in combination with flavopiridol resulted in only 7.3 ± 1.6% and 7.7 ± 0.7% apoptosis respectively (data not

shown), very similar to the amounts observed with flavopiridol alone (7.2%). These concentrations of PBOX-6 and -15 alone were insufficient to produce any cell cycle effects in PC3 cells and indicated that the G_2/M arresting properties of PBOXs were required for synergism with flavopiridol. As expected, these concentrations of PBOXs were not deemed by CalcuSyn to be synergistic with flavopiridol (Fig. 5B,C). In fact, median dose analysis actually graded these combinations as antagonistic (CI value>1) concluding that they produced a smaller than expected additive effect. However, this is because the program did not take into account the fact that the amount of apoptosis (1.3% and 0.9%) generated at these concentrations of PBOX-6 and -15 was merely background levels of apoptosis also found in vehicle-treated controls.

Discussion

The treatment of hormone-refractory advanced prostate cancer poses a significant challenge for clinicians. Until recently hormone-refractory prostate cancer was considered chemotherapy-refractory with the standard treatment of mitoxantrone plus prednisone merely providing palliative benefits. This theory was refuted in 2004 by clinical trials demonstrating that docetaxel plus prednisone (3) or docetaxel plus estramustine (4) significantly improved overall survival compared with mitoxantrone plus prednisone. Hence, the current standard of care for patients with this stage of prostate cancer is systemic chemotherapy with a docetaxel-based regimen (2). Unfortunately, over time, most patients still progress to develop resistance to docetaxel. Much of this resistance is thought to be mediated through expression of the multidrug-resistance transporter, P-glycoprotein or by microtubule alterations (21). In addition to inducing apoptosis in a variety of cancerous cell types (5-9) and impairing tumour growth *in vivo* (11,12), novel pyrrolo-1,5-benzoxazepine (PBOX) compounds possess the added benefit of inducing cytotoxicity in multidrug-resistant cancer cells expressing P-glycoprotein with similar potency as in p-glycoprotein-negative cancer cells (14). Furthermore, they appear to induce tubulin depolymerisation by binding to a novel as yet uncharacterized site on tubulin (15). To date the effect of PBOX compounds on prostate cancer has not been examined. Hence, in this study we wished to perform some initial *in vitro* experiments to determine if PBOXs may have any effect on advanced hormone-refractory prostate adenocarcinoma cells.

Herein, we report that PBOXs are capable of reducing cell proliferation of prostate cancer PC3 cells with IC_{50} values of 5.8 μM for parent compound PBOX-6 and 0.37 μM for its more potent analogue, PBOX-15. These values are within a similar range (1-6 μM and 0.2-0.4 μM for PBOX-6 and -15 respectively) to those observed in other cell lines such as CML K562

cells, promyelocytic leukaemia HL60 cells, ovarian carcinoma A2780 cells and a range of mammary carcinoma cell lines (10, 11, 14, 16). This reduction in PC3 cell proliferation was mediated through a significant G_2/M arrest which occurred concurrently with depolymerisation of the microtubular network. G_2/M arrest was followed by a modest though significant amount of apoptosis. PBOX-15 induced slightly less apoptosis than PBOX-6 relating to a greater number of cells remaining in the G_2/M phase and suggested PBOX-15 produced a slightly more sustained G_2/M block than PBOX-6 at 72 h. Progression to apoptosis induced by PBOXs in PC3 cells seemed to follow patterns previously observed in other cell types including CML K562 cells, HeLa cells and A2780 cells which also exhibited a sustained G_2/M arrest followed by some apoptosis. In contrast, cell types such as HL60 and breast SK-BR-3 undergo only transient arrest followed by massive amounts of apoptosis (10, 14, 16).

In order to delineate some of the mechanisms that may be involved in the response of PC3 cells to PBOX treatment, we examined some proteins which influence cell cycle arrest and apoptosis, namely, BubR1, Bcl-2 and Bcl-x_L. Firstly, we examined the mitotic spindle checkpoint protein BubR1, which monitors tension across attached kinetochores and initiates mitotic arrest in response to loss of microtubule tension (22). We have previously reported that expression of BubR1 directly relates to the length of time a cell remains in G_2/M, given that cells which expressed very little BubR1 such as HL60s underwent a very rapid transition from G_2/M to apoptosis while cells such as K562s which highly expressed BubR1 remained arrested in G_2/M much longer (16). Our DNA content analysis data demonstrated that PC3 cells remained in G_2/M for quite a sustained period with some cells still remaining arrested after 72 h, therefore, we decided to investigate if BubR1 is associated with this sustained arrest. Indeed, we found that initially BubR1 was hyperphosphorylated in PC3 cells treated

with PBOX-6 or -15. Hyperphosphorylation of BubR1 is indicative of an active mitotic checkpoint (23). Subsequently, expression of BubR1 declined at later time points corresponding to a decrease in the amount of cells undergoing G_2/M arrest and an increase in apoptosis, indicating that the phosphorylation status of BubR1 in PC3 cells directly correlated to the quantities of G_2/M arrest found in these cells. Therefore, we postulate that the expression of BubR1 in PC3 cells and its subsequent phosphorylation is likely to have contributed to the sustained arrest observed.

As microtubules have been shown to interact with mitochondria, it is thought that the mitochondria and their associated proteins connect microtubular insult to the apoptotic machinery (24). Mitochondrial anti-apoptotic proteins Bcl-2 and Bcl-x_L are important in controlling apoptosis by preventing the release of mitochondrial pro-apoptotic factors, critical for cell death via the intrinsic apoptotic pathway. Over-expression of Bcl-2 or Bcl-x_L is a common feature in many malignancies and confers resistance to a range of cytotoxic agents (25). Specifically, over-expression of Bcl-2 (26) and Bcl-x_L (27) are implicated in the development of hormone-resistance in prostate cancer cells. Over-expression of these proteins seems to block the events downstream of mitotic arrest that lead to apoptosis since Bcl-2 or Bcl-x_L over-expression suppresses the apoptotic response of microtubule-targeting agents without affecting their actions on microtubules or their ability to induce G_2/M arrest (28, 29). We have previously demonstrated that PBOX-6 induces apoptosis in leukaemia cells over-expressing Bcl-2 with similar potency as in leukaemia cells expressing normal levels of Bcl-2 (8). During this study we found that both PBOX-6 and -15 induced a transient phosphorylation of Bcl-2 and Bcl-x_L which correlated to initiation of G_2/M arrest in PC3 cells. Indeed, phosphorylation of Bcl-2 is considered a marker of M phase since it is also phosphorylated at G_2/M phase of normally cycling cells (30). Following phosphorylation,

expression of Bcl-2 and Bcl-x_L diminished. These results adhere to an expected pattern of Bcl-2 and BCL-x_L phosphorylation and inactivation since we have previously observed a similar effect in leukaemia cells whereby we found that phosphorylation and inactivation of Bcl-2 and Bcl-x_L by the stress-activated protein kinase JNK (c-Jun N-terminal kinase) preceded PBOX-6-induced apoptosis (8). Furthermore, other studies have shown that phosphorylation of Bcl-2 is specifically induced by microtubule-targeting agents and not with DNA-damaging drugs and this phosphorylation is a prerequisite to inactivation/degradation of Bcl-2 (24).

Despite the fact that the PBOX-6 and -15 induced a significant amount of apoptosis in PC3 cells after 72 h, a number of cells still remained resistant to apoptosis. This phenomenon was associated with sustained G_2/M arrest. Progression at the G_2-M transition is regulated by a complex consisting of a regulatory subunit, cyclin B1 and a catalytic subunit cyclin-dependent kinase-1 (CDK-1) (31). Activation of the mitotic spindle checkpoint blocks the degradation of cyclin B1 and thus results in sustained activation of the cyclin B1/CDK-1 complex leading to G_2/M arrest (24). We postulated that this sustained activation of the CDK1/cyclin B1 complex may play a role in the prolonged arrest observed in PC3 cells treated with PBOXs. Hence, we hypothesised that by reducing the activities of CDK-1 after the cells have already undergone G_2/M arrest, we may increase the rate of apoptosis generated by PBOXs in PC3 cells. Indeed, we have previously reported that inhibition of CDK-1 by its inhibitor, flavopiridol enhanced PBOX-induced apoptosis in CML cells by accelerating exit from G_2/M (32). Sequential treatment with flavopiridol is also reported to enhance effects of docetaxel in patients with advanced solid tumours (33). Therefore, we decided to investigate if flavopiridol was capable of enhancing PBOX-induced apoptosis in PC3 cells. As in CML cells, we found that flavopirdol administered as a sequential treatment

after PBOX-induced G_2/M arrest, synergistically enhanced apoptosis in PC3 cells compared to treatment with PBOXs alone.

Our results indicate that PC3 cells seem to respond to PBOX treatment in a similar manner to CML K562 cells, whereby they induce a sustained G_2/M follow by a degree of apoptosis with many cells still remaining in G_2/M. In contrast, other cells types such as HL60s undergo a transient G_2/M arrest followed by almost complete apoptosis in response to PBOXs. Since PC3 cells respond to PBOXs in a similar manner to CML K562 cells, it would be interesting to see if PBOXs would also behave similarly in an *in vivo* prostate tumour model to our *in vivo* CML mouse model in which tumour growth was successfully impaired by PBOX treatment (12). It would also be interesting to examine other combinations which may potentiate PBOX-induced apoptosis. For example, since skeletal metastases are commonly associated with advanced prostate cancer it may be useful to investigate a combination between PBOXs and with one of the anti-resorptive bisphosphonates such as zoledronic acid. This agent has already been found to enhance docetaxel treatment in PC3 cells (34) and along with its ability to palliate skeletal symptoms, it is postulated to reduce the incidence of skeletal events in patients with prostate cancer through its anti-resorptive properties (35) and also have a direct effect on the prostate cancer cells (36). Additionally, our group is currently investigating the use of PBOXs as radiosensitizing agents for advanced but localised tumours, since G_2/M arrest and phosphorylation of Bcl-2 and Bcl-x_L by PBOXs may reduce the apoptotic threshold of tumour cells and hence render them more susceptible to death stimuli such as radiation. It may also prove beneficial to assess the effects of PBOXs alone and in combination with other compounds specifically in docetaxel-resistant prostate cancer cells.

In summary, our results to date indicated that PBOX compounds either alone or in combination may prove useful in the treatment of advanced hormone-refractory prostate cancer.

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Figures

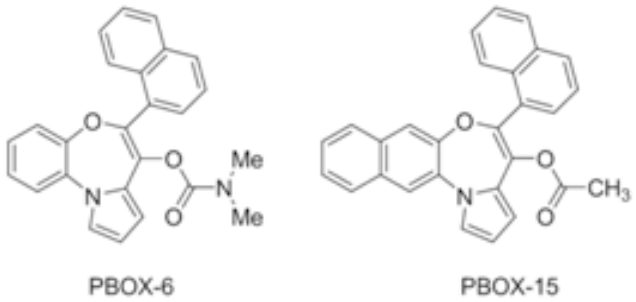

Figure 1. Chemical structures of pyrrolo-1,5-benzoxazepines PBOX-6 and PBOX-15

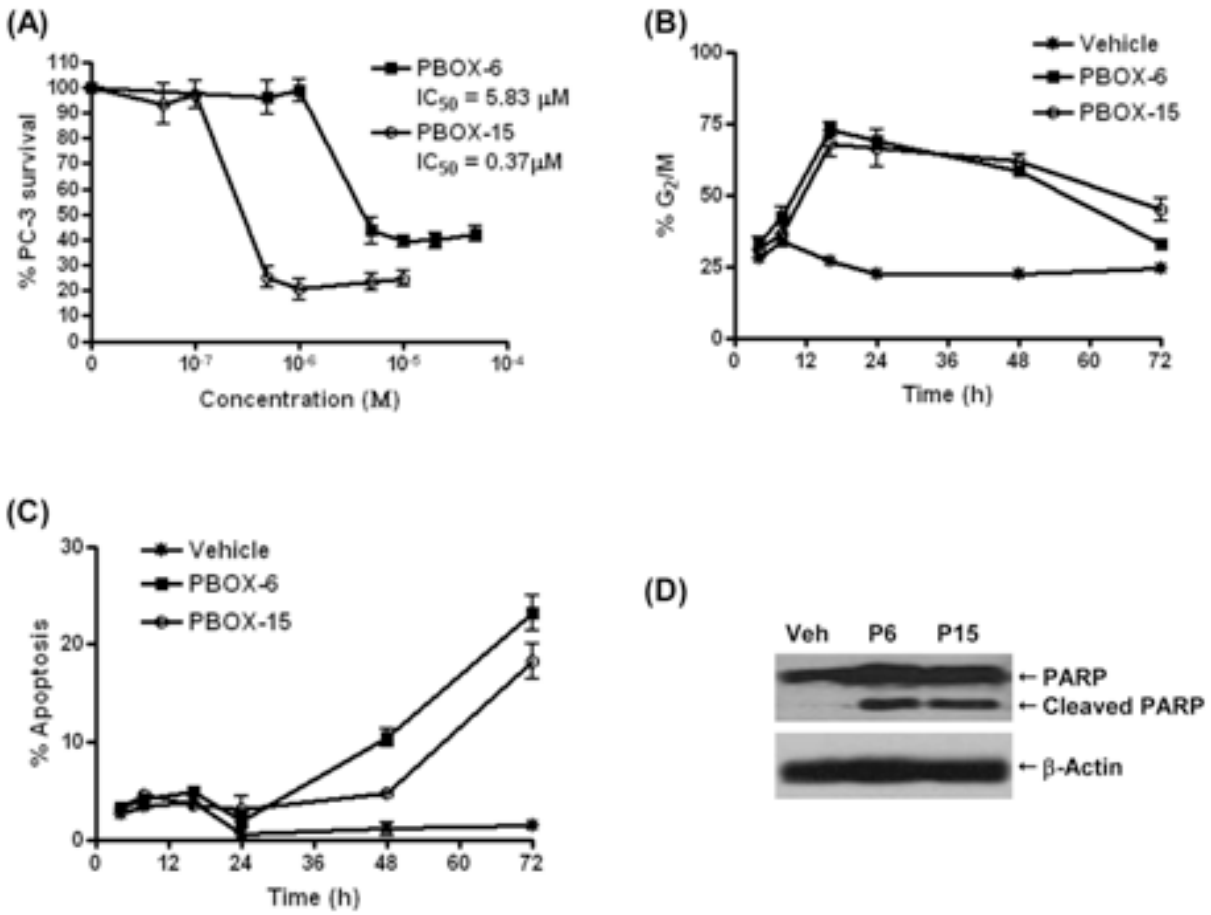

Figure 2. PBOX-6 and PBOX-15 reduced proliferation and induced G_2/M arrest followed by apoptosis of hormone-refractory prostate carcinoma PC3 cells.

(A) PC3 cells were seeded onto 96-well plates and treated in triplicate with vehicle alone (1% (v/v) ethanol) or a range of concentrations of PBOX-6 or PBOX-15 for 72 h. The cells were then incubated in 10% (v/v) AlamarBlue™ and its reduction to a fluorescent state measured at excitation 544 nm and emission 590 nm using a multi-well fluorimeter. The results were expressed as the percentage cell proliferation relative to vehicle-treated control cells (100%). Values represent the mean ± the S.E.M for three separate experiments

(B,C) PC3 cells were treated with vehicle (0.5% (v/v) ethanol), PBOX-6 (10 µM) or PBOX-15 (0.5 µM) for up to 72 h, fixed in ethanol, stained with propidium iodide and DNA content assessed by flow cytometry. Analysis of data was performed using the computer program

CellQuest. Cells in the subG_0/G_1 phase (<2N DNA) were deemed apoptotic (C), while cells with 4N quantities of DNA were considered to be in the G_2/M phase of the cell cycle (B). Values represent the mean ± S.E.M for three independent experiments.

(D) PC3 cells were treated with vehicle (0.5% (v/v) ethanol), PBOX-6 (10 μM) or PBOX-15 (0.5 μM) for 72 h. PARP cleavage was assessed by Western blot using monoclonal antibodies generated against PARP or loading control β-actin, followed by a HRP-conjugated anti-mouse secondary antibody. Blots are representative of three independent experiments.

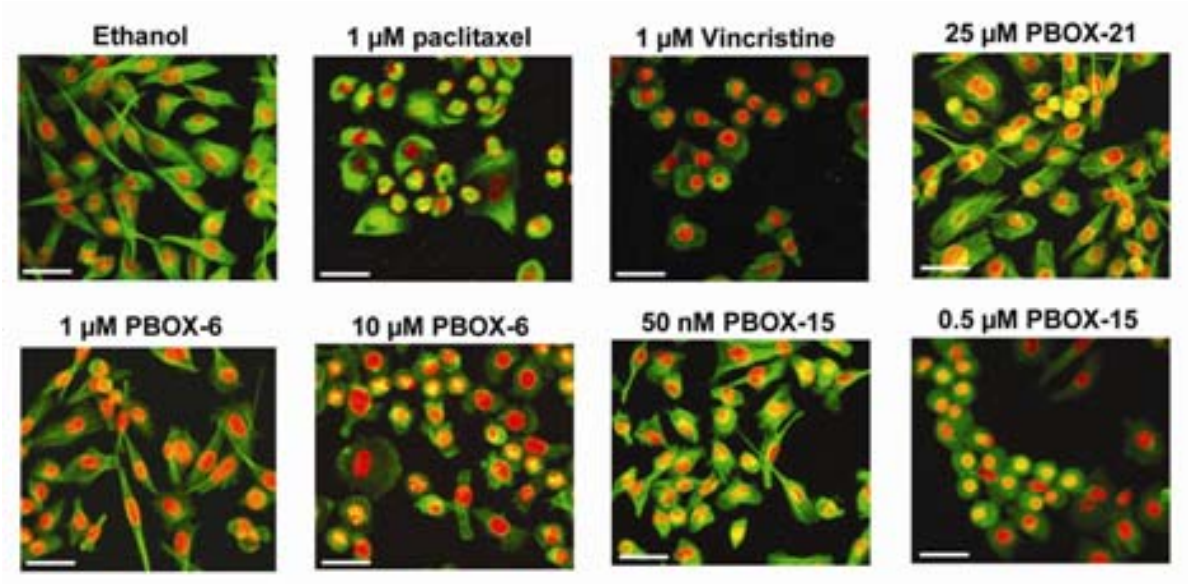

Figure 3. PBOX-6 and PBOX-15 resulted in disruption to the organisation of the microtubule network in PC3 cells.

PC3 cells grown on 4-chamber glass slides were treated with the indicated doses of vehicle (0.5% (v/v) EtOH), paclitaxel, vincristine, PBOX-21, PBOX-6 or PBOX-15 for 16 h. The cells were then fixed in methanol, incubated with a monoclonal anti-α-tubulin antibody, followed by a FITC-conjugated anti-mouse antibody and then briefly stained with propidium iodide. The organisation of the microtubule network (green) and the cellular DNA (red) was visualised using a fluorescent microscope at a magnification of 600X [bar = 40 μm]. Photographs illustrated are representative of three independent experiments

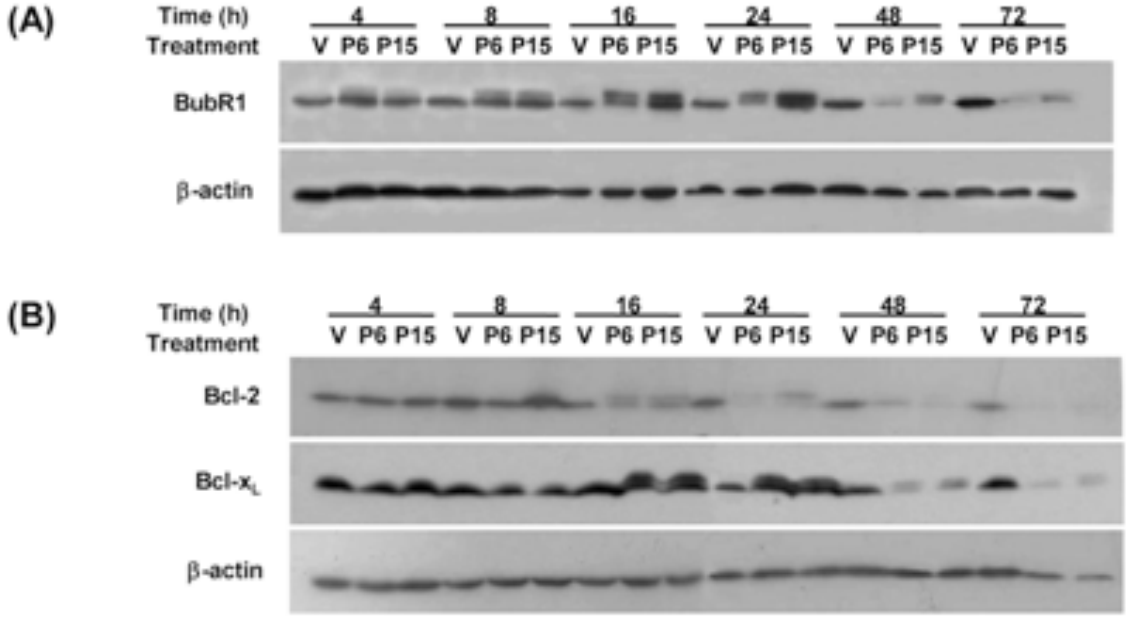

Figure 4. PBOX-6 and PBOX-15 induced phosphorylation followed by degradation of mitotic spindle checkpoint protein BubR1 and anti-apoptotic proteins Bcl-2 and Bcl-x_L in PC3 cells.

PC3 cells were treated with vehicle (0.5% (v/v) EtOH) (veh), 10 μM PBOX-6 (P6) or 0.5 μM PBOX-15 (P15) for the indicated times. Protein phosphorylation was then assessed by Western blotting using primary antibodies for BubR1 (A), Bcl-2 (B), Bcl-x_L(B) or β-actin (loading control), followed by a HRP-conjugated secondary antibody. Blots are representative of three independent experiments.

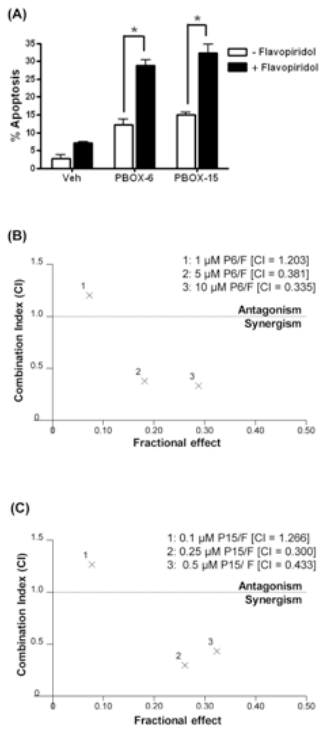

Figure 5. The CDK-1 inhibitor, flavopiridol, synergistically enhanced PBOX-induced apoptosis in PC3 cells.

PC3 cells were treated for 18 h with vehicle (0.5% (v/v) ethanol), PBOX-6 (1-5 μM) or PBOX-15 (0.1-0.5 μM) followed by treatment for 24 h in either fresh media (as a control) or media containing flavopiridol (0.1-0.5 μM). The cells were then fixed in ethanol, stained with propidium iodide and DNA content assessed by flow cytometry using CellQuest. Cells in the subG_0/G_1 phase (<2N DNA) were deemed apoptotic. Apoptosis induced by PBOX-6 (10 μM) or PBOX-15 (0.5 μM) alone or the same treatments followed subsequently by exposure to flavopiridol (0.5 μM) was compared (A). Values represent the mean ± S.E.M for three independent experiments (*$P < 0.05$). Median dose effect analysis was carried out using the software program CalcuSyn. Combination index (CI) values for varying concentrations of PBOX-6 (P6) (B) or PBOX-15 (P15) (C) in combination with 0.5 μM flavopiridol (F) were generated. A CI value of less than one represents synergism while a CI value greater than one represents antagonism.